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13. ABSTRACT (Maximum 200 Words)

Androgen Receptor Trapped clone-27 (ART-27) is a recently identified androgen receptor (AR) N-terminal coactivator. To investigate the potential biological significnance of ART-27 to AR-dependent process such as prostate cancer and androgen insensitivity syndrome (AIS), we examined thirteen naturally occurring AR N-terminal amino acid substitutions identified in prostate cancer and AIS for their ability to affect the AR transcriptional response to ART-27. Of these mutants, AR P340L, a somatic alteration associated with prostate cancer, showed a reduced capacity to enhance ART-27 mediated AR-transcriptional activation, whereas its response to members of the p160 class of steroid receptor coactivators was unaffected. Interestingly, more ART-27 appears to associate with the AR P340L substitution as compared to the wild type receptor. Therefore, AR P340L interacts more avidly with ART-27, yet paradoxically decreases AR transcriptional activity, perhaps as a consequence of inappropriate Ar-coactivator binding. This may represent a novel mechanism of pathogenesis whereby increased AR-coactivator association negatively regulates AR activity and biological response. Given that AR mutations in prostate cancer, like AR P340L, are likely to confer a growth advantage to the cell, our results support a role for ART-27 in AR-dependent growth suppression, rather than receptor-mediated cellular proliferation.

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Introduction

The Androgen receptor (AR) is a hormone-dependent transcription factor involved in the regulation of both normal and malignant prostate cell growth. However, the precise mechanisms by which AR regulates normal prostate development and initiates prostate cancer have yet to be elucidated. It is believed that co-factors (coactivators and corepressors) that interact with the AR and modulate its activity play an important role in these processes. ART-27, as a newly identified AR coactivator, its function in normal prostate development and prostate cancer need yet to be elucidated. Modulation ART-27 levels in vivo such as knocking out ART-27 in the mouse will be our ultimate answer to these questions. Meanwhile recent studies have suggested that ART-27 may confer AR-dependent growth suppression and differentiation, if so, any alteration in ART27:AR interaction with those N-terminal mutated AR identified in some prostate cancer patients, will provide important genetic evidence for the role of ART27 in human disease, such as prostate cancer.

Body

Task 1 Determine if AR N-terminal mutations that have been identified in prostate and androgen insensitivity syndrome (AIS) affect ART-27 binding or function.

This task has been carried out and finished. Detailed work can reference appended manuscript, which is in the process of submission.

Task 2 To create ART-27 null mice by targeted mutagenesis and assess the consequences of lack of ART-27 expression on prostate development.

In my proposal, the preliminary data included the already-made ART-27 targeting vector and two correctly targeted ART-27-/+ ES cell clone. I have used the ES clones to inject blastocysts and have them transplanted into pseudopregnant female mice. However, recent examination of this genomic DNA, which I generated to target to, revealed that it was a pseudogene because its predicted transcribed sequence had an early stop codon. Searching the newly updated genebank database confirmed this finding. The true ART-27 gene maps to chromosome X, like in humans, not chromosome 15 which I originally thought so.
As this part of job has to be started from the very beginning, one-year term won't be enough to finish this task; I would like to change my future work to ART-27 promoter studies.
Histoimmunochemistry studies have just shown that in normal adult human prostate, ART-27 is expressed in luminal epithelial cells, but not in the stroma. During prostate development, ART-27 is expressed in differentiated luminal epithelial cells, but not in their undifferentiated precursors. Further, ART-27 expression levels are negligible in human prostate cancer and increased expression of ART-27 in the androgen-responsive LNCaP prostate cancer cell line inhibits androgen-mediated cellular proliferation. All these suggest a possible role for ART-27 in AR-mediated growth suppression and differentiation of the prostate epithelium. It is conceivable that understanding how ART-27 is regulated on transcription level will help us to design a new target for prostate cancer treatment.

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Key research Accomplishments

1. Constructed a series of point mutations in the AR N-terminus that correspond to alterations observed in AIS and prostate cancer by site directed mutagenesis.

2. Analyzed their effects on AR-mediated transcriptional activation and expression in cultured cells.

3. Analyzed their interaction with ART-27.

4. Analyzed their effects on ART-27-dependent receptor transcriptional activation.

Reportable Outcomes

A manuscript in submission (see appendex);

A poster presentation in 2004 Keystone Symposium "Nuclear Receptor:Steroid Sisters" , Denver, CO

Conclusions

It has been proposed that aberrant interactions between the androgen receptor (AR) and its coregulators contribute to prostate cancer; however, evidence linking abnormal receptor:cofactor interaction to disease is scant. Here We found that an AR somatic alteration in a prostate cancer (AR P340L) displays reduced response to ART-27 coactivation relative to the wild type AR, whereas its response to the p160 class of coactivators was not affected. Despite the fact that AR P340L shows decreased transcriptional activation in response to ART-27, more ART-27 associates with AR P340L as compared to the wild type receptor. Thus, P340L promotes a more avid AR:ART-27 interaction, suggesting that aberrant AR-coactivator association interferes with normal ART-27 coactivator function resulting in suppression of AR activity. This represents a novel mechanism of pathogenesis whereby an AR mutation reduces AR susceptibility to its coactivator ART-27, which normally suppresses cellular proliferation, and underscores the importance of loss of ART-27 in oncogenesis.

Further work providing convincing evidence of the functional relevance to tumorigenesis of the P340L AR mutation in a physiologically relevant model for prostate cancer will be greatly helpful.

An androgen receptor mutation identified in prostate cancer displays

aberrant ART-27 coactivator function

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Running title: AR mutants affecting ART-27 activity

Significance

It has been proposed that aberrant interactions between the androgen receptor (AR) and its coregulators contribute to prostate cancer; however, evidence linking abnormal receptor:cofactor interaction to disease is scant. Here we demonstrate that an AR somatic mutation identified in a patient with prostate cancer (AR P340L) shows abnormal interaction and diminished transcriptional response to ART-27, an AR N-terminal coactivator. Our findings suggest that the AR P340L mutant facilitates prostate cancer progression by evading the normal "growth suppressive" function of the AR coactivator ART-27. This represents a novel mechanism of pathogenesis whereby an AR mutation reduces AR susceptibility to its coactivator ART-27, which normally suppresses cellular proliferation, and underscores the importance of loss of ART-27 in oncogenesis.

Summary

The Androgen Receptor Trapped clone-27 (ART-27) is a recently identified AR N-terminal coactivator that is associated with AR-mediated growth inhibition. We found that an AR somatic alteration in a prostate cancer (AR P340L) displays reduced response to ART-27 coactivation relative to the wild type AR, whereas its response to the p160 class of coactivators was not affected. Despite the fact that AR P340L shows decreased transcriptional activation in response to ART-27, more ART-27 associates with AR P340L as compared to the wild type receptor. Thus, P340L promotes a more avid AR:ART-27 interaction, suggesting that aberrant AR-coactivator association interferes with normal ART-27 coactivator function resulting in suppression of AR activity.

Introduction

The androgen receptor (AR) is a transcriptional regulatory protein that transduces the signaling information conveyed by androgens (Gelmann, 2002). Upon androgen binding, the hormone-AR complex enters the nucleus, associates with specific DNA sequences and modulates transcription initiation from nearby promoters (Jenster et al., 1991). Activation of AR is essential for the maintenance of the prostate gland in adult males; in the absence of androgens, the prostate shrinks to a rudimentary form. For this reason, the use of anti-androgens remains a mainstay of prostate cancer treatment (Culig et al., 2003).

The transcriptional activation functions (AFs) of AR (Chamberlain et al., 1996; Jenster et al., 1995) represent surfaces capable of interaction with general transcription factors and additional transcriptional regulatory factors termed coactivators. Coactivators have been identified that interact with the AR N-terminal AF-1 and the C-terminal AF-2 region to enhance AR-dependent gene transcription (He and Wilson, 2002; Heinlein and Chang, 2002). AR also interacts with the general transcription factor TFIIF (Reid et al., 2002b) as well as the Cdk-activating kinase of TFIIH (Lee et al., 2000).

Androgen Receptor Trapped clone-27 (ART-27) was identified in our laboratory as an AR N-terminal coactivator (Markus et al., 2002). Recently, we have shown that in normal adult human prostate, ART-27 is expressed in luminal epithelial cells, but not in the stroma (Taneja et al., 2004). During prostate development, ART-27 is expressed in differentiated luminal epithelial cells, but not in their undifferentiated precursors. Further, ART-27 expression levels are negligible in human prostate cancer and increased expression of ART-27 in the androgen-responsive LNCaP prostate cancer cell line inhibits

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androgen-mediated cellular proliferation, suggesting a role for ART-27 in AR-mediated growth suppression and differentiation of the prostate epithelium (Taneja et al., 2004).

AR mutations have been identified in prostate cancers, and may confer a growth advantage to the cell (Buchanan et al., 2001). In principle, AR somatic alterations associated with prostate cancer may represent gain-of-function mutations that enhance AR interaction with coactivators involved in cell proliferation. Alternatively, if coactivators such as ART-27 confer AR-dependent growth suppression and differentiation, then the function of ART-27 may be reduced in certain receptor mutations isolated from prostate cancer patients.

A group of naturally occurring mutations are also found in patients with Androgen Insensitivity Syndrome (AIS), a condition whereby individuals are genetically male, yet phenotypically female as a result of defective AR signaling (Brinkmann, 2001; Gottlieb et al., 1999). The AR N-terminal mutations that correlate with AIS are presumed to produce AR loss-of-function mutants perhaps as a consequence of disrupted AR-coactivator interactions. Here we analyze naturally occurring AR mutations identified in prostate cancer and AIS for their ability to functionally interact with ART-27.

Results

To examine the physiological contribution of ART-27 to AR-dependent

processes, we tested a set of naturally occurring AR N-terminal mutations identified in

prostate cancer and AIS for their effect on the AR transcriptional response to ART-27

(Figure 1A). It is conceivable that these AR mutations induce changes in the receptor

structure, such that unstructured regions would adopt structural features or alternatively,

that structured domains would be disrupted. Therefore, we analyzed the ability of the AR

mutations to alter AR structure prior to embarking on the functional analysis. Initially,

we analyzed the secondary structure of the wild type AR N-terminus from a multiple

alignment among ARs from different species (Figure1A). This approach can achieve

>75% accuracy (Frishman and Argos, 1996; Frishman and Argos, 1997) and revealed 16

regions with predicted structural features, although the majority of the AR N-terminus

appears unstructured, which is in agreement with biophysical studies (Reid et al., 2002a).

Of the AR mutations analyzed, the majority had no apparent effect on the secondary

structure of the AR N-terminus (not shown). The two mutations that did change the

predicted structure of the AR N-terminus were AR E2K, a germ line mutation identified a

patient with partial AIS (Choong et al., 1996), and AR P340L, a somatic mutation

identified in a localized prostate cancer (Castagnaro et al., 1993). Analysis of the low-

energy conformations (within 5 kcal/mol) accumulated during extensive peptide

simulations of the AR residues 1-25 shows that the first ten residues form an α-helical

fold in the E2K mutant, whereas this region in largely unstructured in the wild type AR

(Figure 1B and C). Interestingly, the tertiary structure simulation for the AR 331-355

region shows a dramatic preference for wild type peptide to adopt an α-helical fold at its

C-terminus between residues 341-355, whereas the P340L mutation folds into a α-helix near the N-terminus flanked by residues 331-345 (Figure 1D and E). Therefore, the E2K and P340L mutations generate new structural elements, suggesting that they may represent functionally relevant alterations.

Next we examined the effect of ART-27 overexpression on wild type and mutant AR transcriptional activation (Figure 2A). The transcriptional activity of the wild type AR was increased roughly three-fold by ART-27 overexpression (Figure 2A). Interestingly, the AR mutations P340L and E2K displayed reduced ART-27-dependent receptor transcriptional enhancement, whereas the other alterations appeared capable of increased activation in response to ART-27. Thus, the AR mutants E2K and P340L appear to have a reduced capacity to utilize ART-27 as a coactivator.

In addition to measuring total hormone-dependent AR activity, we also compared the "fold-induction" or the AR transcriptional response to ligand in the presence and absence of ART-27 (Figure 2C). The relative fold-induction of AR in response to ART-27 is constant over a range of AR concentrations and, therefore, is a valid means of comparison among the receptor mutants that vary in expression (Supplemental Data Figure 1). The AR alterations E2K and P340L, maintain roughly the same fold-activation in response to ligand as the wild type AR, but showed a decreased response to overexpressed ART-27 (Figure 2C). The AR E2K mutation, originally identified from an AIS patient, has been previously shown to exhibit reduced receptor expression as a result of inefficient translation (Choong et al., 1996). Consistent with this characterization, we also observe decreased protein expression of AR E2K relative to wild type AR in either the absence or presence of ART-27 (Figure 2B). In contrast, AR P340L exhibits steady

state protein levels higher than the wild type AR regardless of the level of ART-27 despite a diminished response. The same effect was also observed with the ARR_3-luciferase reporter from the Androgen Responsive Region (ARR) of the rat probasin promoter (Kasper et al., 1999) (Figure 2D) and from the synthetic TAT_3-luciferase (not shown), indicating that the effect of the E2K and P340L substitutions on the receptor transcriptional response to ART-27 is evident at distinct AREs and promoter elements. Increased ART-27, AR or hormone concentration does not compensate for the defect (Supplemental Data Figures 2A-C).

To determine if these AR mutants show decreased responsiveness to coactivators other than ART-27, cells were transfected with wild type AR, AR E2K and AR P340L along with ART-27 or the p160 coactivators, GRIP-1 or SRC-1 (Stallcup et al., 2003; Xu and Li, 2003). As before, the AR P340L substitution showed a diminished capacity to respond to ART-27 relative to the wild type AR: a 50-fold-induction of wild type AR to ART-27 *versus* a 29-fold-induction of AR P340L to ART-27 (Figure 3). In contrast, the AR transcriptional response to SRC-1or GRIP-1 was not affected by the AR P340L substitution relative to the wild type receptor: a 33-fold-induction of wild type AR *versus* a 31-fold-induction of AR P340L to SRC-1 and a 37-fold-induction of wild type AR *versus* a 36-fold response of AR P340L for GRIP-1. Analysis of the AR E2K mutation again showed a reduced ability to respond to ART-27 (Figure 3). Surprisingly, AR E2K shows a significant increase in transcriptional activity in response to SRC-1 (Figure 3). Thus, the E2K substitution may promote AR:SRC-1 interaction. Our findings indicate that the E2K and P340L mutations affect AR functional interactions with ART-27.

We next tested the AR mutants for physical interaction with ART-27 by co-immunoprecipitation. HeLa cells were transfected with a HA-tagged version of ART-27 along with expression vectors for either the wild type AR, AR E2K or AR P340L. Cells were treated with hormone and lysed under conditions that preserve the interaction between AR and ART-27. AR immunoprecipitates were analyzed by immunoblotting with an antibody to the HA moiety on ART-27. As seen in Figure 4A, ART-27 was co-immunoprecipitated with both wild type AR, AR E2K, and AR P340L. About half the level of ART-27 was detected in association with AR E2K. Thus, the AR E2K alteration reduces interaction with ART-27. In contrast, AR P340L showed an increase in its association with ART-27, despite its inability to enhance AR transcription. To investigate whether the increased ART-27 associated with AR P340L mutant might reflect a tighter binding, reciprocal immunoprecipitation experiments between AR and ART-27 were performed under more stringent conditions. Under these circumstances, virtually no ART-27 is detected in association with the wild type AR (Figure 4B). In contrast, ART-27 is readily detected in association with the AR P340L substitution, indicating that the interaction of AR P340L with ART-27 is more stable than that of the wild type receptor. This finding suggests that the AR P340L mutant is binding inappropriately to ART-27 and thus fails to affect AR-dependent transcriptional activation.

Discussion

Although AR plays a role in normal and malignant prostate cell function, its impact on the etiology of prostate cancer is not clear (Nelson et al., 2003). One

possibility is that the altered expression of AR coactivators may promote tumorigenesis. A recent report indicates that recurrent prostate cancers express higher than normal levels of the p160 coactivators, TIF-2/GRIP-1 and SRC-1 (Gregory et al., 2001). In addition, the expression levels of multiple AR coactivators varied between normal and malignant prostate tissue samples (Fujimoto et al., 2001; Li et al., 2002; Mestayer et al., 2003). Further, our group has found that ART-27 protein levels are decreased in prostate cancer (Taneja et al., 2004). Our findings show that aberrant interactions between AR and the AR N-terminal coactivator ART-27 may contribute to diseases related to AR activity, such as prostate cancer and AIS.

Previous studies have shown that the AR E2K mutation decreases receptor translation, resulting in lower steady state AR levels (Choong et al., 1996). Our results also indicate that this mutant shows a diminished interaction and transcriptional response to ART-27. As the AR E2K mutation is outside of the ART-27 binding region (Figure 1A) and induces a local conformational change (Figure 1D), we suggest that it may affect the global conformation of the receptor, which reduces ART-27 binding. Consistent with this notion is the unexpected finding that the AR E2K displays an enhanced transcriptional response to SRC-1. Therefore, the AR E2K mutant may acquire a conformation optimized for SRC-1 interaction at the expense of ART-27 binding, which may be an important determinant of the AIS phenotype.

Our results also indicate that the ability of ART-27 to function as an AR coactivator is greatly decreased by the P340L substitution. While the expectation was that this reduced activity is a result of diminished ART-27 binding to the receptor, this is not the case (Figure 4). Instead, our findings demonstrate that the AR P340L mutant

associates more avidly with ART-27. In principle, increased ART-27 binding to AR could strengthen the binding of an inhibitor or weaken the association of an activator. In support of this latter idea, AR P340L lies near a stretch of amino acids that has been shown to interact with THIIF, a component of the basal transcription machinery consisting of two subunits, RAP74 and RAP30 (Reid et al., 2003; Reid et al., 2002b). Elegant work from the McEwan lab has revealed that RAP74 interacts with AR at multiple sites including two motifs (PSTLSL) located between residues 159-164 and 340-345 in the AR N-terminus (Reid et al., 2002b). One possibility is that the AR P340L mutation creates a new surface for ART-27 binding, and eliminates a motif existing in the wild type AR for cofactor binding, which his consistent with the structure prediction (Figure 1E). This could explain the tendency of AR P340L mutant to exhibit increased ART-27 binding, but decrease AR activity. Interestingly, a search of the AR N-terminus with a 15 amino acid segment between AR residues 334-348 where proline 340 has been substituted with a leucine revealed a conserved motif, T # x LL, where # is any hydrophobic amino acid and x is any amino acid, within AF-1a residues 186-193. We speculate that this might serve as an ART-27 binding site, analogous to the LxxLL motif for AF-2 coactivators.

Another possibility is that ART-27 functions as a chaperone to help "load" TFIIF (or another factor) onto the receptor or maintain AR in a conformation competent for cofactor binding. Once this is accomplished, ART-27 would then dissociate from the receptor. In the AR P340L mutant, however, ART-27 would be unable to correctly place the cofactor onto the receptor or promote a receptor conformation compatible with cofactor binding and would neither dissociate nor coactivate. This idea is not

inconceivable since ART-27 shows homology to prefoldins, which are small molecular weight proteins that assemble into molecular chaperone complexes to affect protein folding. An unconventional prefoldin complex has been recently described that controls a transcription program in response to nutrient deprivation (Gstaiger et al., 2003). Further experiments will distinguish among these possibilities.

Our recent studies indicate that ART-27 expression levels are negligible in prostate cancer as opposed to normal prostate (Taneja et al., 2004). Further, examination of ART-27 protein expression in prostate development demonstrates that ART-27 is detected only when the prostate gland has proceeded from a solid mass of undifferentiated cells to a stage where differentiated luminal epithelial cells are evident (Taneja et al., 2004). In light of these findings, we suggest that ART-27 plays a role in suppressing prostate cancer development by contributing to the maintenance of a program of AR-mediated differentiation. Thus, the AR P340L mutant would facilitate prostate cancer progression by preventing the normal "growth suppressive" function of ART-27. This may represent a novel mechanism of pathogenesis, whereby an AR mutation acts in dominant negative fashion to reduce the action of ART-27 in maintaining differentiation and further highlights the importance of loss of ART-27 function in oncogenic transformation of prostate epithelial cells.

Experimental Procedures

Plasmid construction   The AR mutants were generated by QuickChange site-directed mutagenesis system (Stratagene) using the oligonucleotides described in Table 1 (Supplemental Data) and the wild type pcDNA3:hAR expression plasmid as the template. All mutations were confirmed by DNA sequencing. The p160 expression vectors, pcDNA3-GRIP-1, pCR3-hSRC-1A have been described previously (Rogatsky et al., 2001).

Cell culture and transient transfection   HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories) and 2 mM L-glutamine (Invitrogen). Cells were seeded in a 24-well plate at a density of 3×10^4 or a 6-well plate at a density of 1.5×10^5 in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS. Transfection was performed using Lipofectamine Plus (Invitrogen) according to manufacturer's instructions. For transfection of cells in 24-well plats, each well received 40 ng of the pcDNA3:hAR expression vector, 100 ng of androgen-response reporter plasmid MMTV-luciferase, and 10 ng of CMV-LacZ, together with 100 ng of pcDNA3-HA-ART-27. The total amount of DNA transfected was held constant using the corresponding empty vector. For SRC-1 and GRIP-1, 100 ng of each plasmid was used. For 6-well plates, all plasmid amounts were increased 5-fold. After a 3-hour incubation, the transfection mixtures were removed and the cells were refed with phenol red-free medium. The next day, the indicated amount of R1881 (Perkin-Elmer) or an equal volume of an ethanol vehicle was added. After 24 hours, the cells were washed with phosphate-buffered saline (PBS) and lysed in 1 x luciferase cell

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culture lysis reagent (Promega; Cat No E1500). The cell extracts were analyzed for luciferase activity and the values were normalized to β-galactosidase activity. Luciferase activity was quantified in a reaction mixture containing 15 μl of lysate and 100 μl of luciferase assay reagent (25 mM glycylglycine pH 7.8, 10 mM $MgSO_4$, 1 mM ATP, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol (DTT)), using an LMax microplate reader luminometer and 1mM D-luciferin as substrate. Parallel sets of cells were analyzed for AR protein expression.

Coimmunoprecipitation   For each 10 cm dish of HeLa cells, 10 μg AR or AR mutants was cotransfected with 10 μg HA-ART-27. Three-hours post-transfection, the cells were treated with 100 nM R1881 or ethanol vehicle for 16 hours, washed with cold PBS, scraped and collected into a 15 ml conical tube by low speed centrifugation. For interactions under low stringency conditions, nuclear extracts were prepared from cell pellets resuspended in 1.5-fold of the packed cell volume (typically 300 μl) of Buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM DTT, 0.2 mM PMSF, and protease inhibitor cocktail). The cells were then lysed by 5 passes through a 24-gauge needle and centrifuged at 14,000 rpm for 5 min at 4°C. The nuclear pellet was resuspended in two-thirds of the original volume (typically 200 μl) of Buffer C (20 mM HEPES pH 7.9, 25% Glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 2 mM DTT, 0.2 mM PMSF, and the protease inhibitor cocktail) and incubated on ice with stirring for 30 min. The nuclear extracts were obtained by centrifugation at 14,000 rpm for 5 min at 4°C.

The coimmunoprecipitation under high stringency conditions was performed by lysing the cells directly on the plate in 300 μl of RIPA buffer (150 mM NaCl, 0.2% SDS,

50 mM Tris, pH 7.4, 1% NP-40, 1% deoxycholate) on ice and centrifugation at 14,000 rpm for 5 min at 4°C.

The total protein concentration was normalized and 10 µg of a mouse monoclonal antibody to HA (Covance) or AR (Santa Cruz Biotechnology Cat. No. sc-7305) was added and incubated overnight at 4°C. After the incubation, 60 µl of a 50% slurry of Protein G Sepharose beads (Amersham Pharmacia Biotech Inc.) was added and incubated for another 2 h at 4°C with rocking. The beads were collected by centrifugation and the immune complexes were washed three times with HEMG buffer (20 mM HEPES, pH 7.9, 12.5 mM $MgCl_2$, 0.2 mM EDTA) for the low stringency precipitation. For the high stringency conditions, the immune complexes were washed three times in low-salt buffer (150 mM NaCl, 20 mM Tris, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100,), twice with high salt buffer (500 mM NaCl, 20 mM Tris, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), and twice with a non-ionic detergent containing wash buffer (250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.1). The beads were resuspended in 2X SDS sample buffer, boiled for 5 min, placed on ice and stored at –20°C.

Immunoblotting   Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon paper (Millipore Corp., Bedford, MA). The membranes were blocked in 5% BSA in Tris-buffered saline pH 7.4 (TBS) at 4°C overnight. The membranes were incubated in the blocking buffer with primary antibody at room temperature for 2-4 h [1:500 of a mouse monoclonal antibody to AR; 1:1000 of a goat polyclonal antibody against actin (Santa Cruz Biotechnology Cat. No.

sc-1616); and 1:1000 of a mouse monoclonal antibody to HA]. The membranes were washed three times for 10 min in TBS/0.1% Triton X-100 and were incubated for 1 h at RT with anti-rabbit, mouse or goat-IgG conjugated to horseradish peroxidase, washed five times for 10 min in TBS/0.1% Triton X-100, followed by TBS and developed with Enhanced chemiluminescence (Amersham Pharmacia Biotech Inc.).

Structure analysis   Secondary structure predictions were based on the Frishman and Argos (Frishman and Argos, 1996) method as implemented in the Internal Coordinates Mechanics (ICM) program. *Peptide simulations*-The 25-residue peptides M1-EVQLGLGRVYPRPPSKTYRGAFQ-N25 and A331-GSSGTLELPSTLSLYKSGALDEA-A355 (with E2K and P340L mutations, respectively) were built into ICM using an all-atom representation according to the ECEPP/3 force field (Nemethy et al., 1992). The total energy also included an entropy term (Abagyan and Totrov, 1994) plus a generalized Born (GB)-based electrostatic term (Totrov, 2004). For each peptide, four parallel independent Biased Probability Monte Carlo (BPMC) (Abagyan and Totrov, 1994) global energy optimizations of all backbone and side chain torsion angles were performed starting from different randomized conformations, and the low-energy conformations were collected in a conformational stack (Abagyan and Argos, 1992). Simulations were terminated after 50 million energy evaluations (which corresponds to ~100,000 random steps followed by ~500 local minimization steps) and redundant conformations were eliminated from the stack.

Figure legends

Figure 1   AR functional domains and predicted structure

A) Schematic diagram of the functional domains of the human AR. Shown are a

ploy-glutamine stretch (Q), activation functions 1a (AF-1a) and 1b (AF-1b) (black), the

DNA binding domain (hatched) and the ligand binding domain (LBD) and activation

function 2 (AF-2). The predicted secondary structure of the AR N-terminus. Thick black

line above the structure alignment depicts the ART-27 binding region. The AR N-

terminal mutations identified in androgen insensitivity (AIS) (top) and prostate cancer

(PCa) (bottom) are shown. A list of AR point mutations can be found at

http://ww2.mcgill.ca/androgendb/ . The secondary structure content for the AR N-

terminus from a multiple alignment comparing AR from eight different species to the

human AR; these include AR from rat (P15207) (Tan et al., 1988), mouse (P19091) (He

et al., 1990), dog (Q9TT90) (Lu et al., 2001), rabbit (P49699) (Krongrad et al., 1995),

lemur (O97776) (Choong et al., 1998), chimpanzee (O97775) (Choong et al., 1998),

macaque (O97952) (Choong et al., 1998), baboon (O97960) (Choong et al., 1998). Black

cylinders represent α-helices; gray arrows are β-sheets and dark gray lines are

unstructured. AR mutants with a single asterisk denote an alteration that was found in

conjunction with another mutation outside the AR N-terminus, whereas those with double

asterisks were identified in a mouse model of prostate cancer. Predicted tertiary

structures of B) wild type AR $_{(1-25)}$ and C) AR $_{(1-25)}$ E2K, D) wild type AR $_{(331-355)}$ and E)

AR $_{(331-355)}$ P340L are shown. The N-terminal regions are in red and C-terminal segments

are shaded blue.

Figure 2   Transcriptional response of AR mutants to ART-27

A) HeLa cells were transiently transfected as described in the Experimental Procedures with the indicated AR expression vector or vector only (VO), MMTV-luciferase reporter gene, and CMV-LacZ, together with pcDNA3-HA:ART-27 (+ART-27) or pcDNA3 (-ART-27). Cells were treated with 100 nM R1881 or an ethanol vehicle and AR transcriptional activation was assayed for luciferase activity, normalized to β-galactosidase activity and expressed as relative light units (RLU). B) Whole cell extracts were prepared from transfected cells and the expression of AR variants was analyzed by Western blotting using an AR or an actin antibody, which serves as a control for loading. C) The relative fold-induction in response to 100 nM R1881of each AR mutant in the absence (gray bars) and presence (black bars) of ART-27. Results shown represent a representative experiment done in duplicate with the error bars representing the range of the mean. The experiment was repeated three times with similar results. D) Effect E2K and P340L mutations on the AR transcriptional response to ART-27 of ARR3.

HeLa cells were transiently transfected with the rat probasin Androgen Responsive Region (ARR) reporter construct, ARR$_3$-TK-luciferase, along with the wild type AR (WT), AR E2K (E2K), AR P340L (P340L) or the empty expression vector (VO) in the absence and presence of ART-27. Luciferase activity was determined in the absence and presence and 100 nM R1881, as described previously. Shown is a representative experiment done in duplicate and repeated three times with similar results.

Figure 3   Specificity of the mutant AR transcriptional response to ART-27

HeLa cells were transiently transfected with the indicated AR derivatives, the MMTV-luciferase reporter gene, and ART-27, GRIP-1 or SRC-1 expression constructs. Cells were treated with 100 nM R1881or an ethanol vehicle and luciferase activity determined as described in Figure 2. Shown is a representative of three independent experiments done in triplicate with the error bars representing the standard error.

Figure 4   Aberrant binding of ART-27 to AR P340L mutation

A) HeLa cells were transfected with HA-ART-27 and either the wild type AR, the AR E2K or AR P340L, treated with 100 nM R1881 for 2 h and were immunoprecipitated with an AR antibody from nuclear extracts under low stringency conditions as described in the Experimental Procedures. Associated proteins were resolved by SDS-PAGE. HA-ART-27-associated with AR was detected by immunoblotting with an antibody against HA. The left panel shows the expression of AR and ART-27 prior to immunoprecipitation (input) and the right panel reveals the ART-27 that was immunoprecipitated with AR (IP). The total amount of AR immunoprecipitated (bottom panel) was used to standardize the amount of associated ART-27 by densitometry, with the wild type AR:ART-27 ratio arbitrarily set as 1. B) HeLa cells were transfected as above and whole cell lysates were prepared in RIPA buffer (high stringency conditions) and reciprocal immunoprecipitations were performed using antibodies against AR or ART-27 (HA). Associated proteins were resolved by SDS-PAGE and revealed by immunoblotting with the corresponding AR or ART-27 (HA) antibody. Shown are representative experiments that were repeated three times with similar results.

Supplemental Data Figure legends

Supplemental Figure 1

Fold-induction of AR by ART-27 is independent of AR protein concentrations

A) HeLa cells in 24-well plates ($3x10^4$ cells/well) were transfected with the indicated

amount of AR in the absence (-);or presence (+) of ART-27 as indicated. Fold-induction

is determined as described in the Experimental Procedures. The relative induction by

ART-27 over the range of AR concentrations examined (fold induction -ART27/+ART-

27) is shown by a black line. Results shown are from a representative of three

experiments done in duplicate and represent the mean. B) Western blot of AR from

transfected cells in the absence (top panel) and presence (bottom panel) of ART-27. Note

that fold-induction of AR by ART-27 is largely independent of the level of AR protein

expression.

Supplemental Figure 2

Diminished response of AR E2K and AR P340L to ART-27 is not restored by

increasing ART-27, AR or hormone levels.

A) Transcriptional response of WT AR, AR E2K and AR P340L to increasing levels of

ART-27. HeLa cells in 6-well plates ($1.5x10^5$ cells/well) were transfected with an

MMTV-luciferase reporter construct (100 ng), the AR derivatives (WT, E2K, P340L or

S334P; 200 ng) and increasing concentrations of ART-27 (0, 0.2, 0.5, 1.0 or 1.5 _g).

Cells were treated with 100 nM R1881 and luciferase activity assayed as described in

Experimental Procedures, normalized to _-galactosidase activity and expressed as relative

light units (RLU). B) Transcriptional response of AR to ART-27 as a function of

receptor concentration. HeLa cells in 24-well plates (3×10^4 cells/well) were transfected with ART-27 (100 ng) and increasing amounts of AR (20, 40, 80, 100 and 160 ng). Cells were treated with 100 nM R1881 and AR transcriptional response from the MMTV-luciferase reporter gene was determined as above. Western blot (bottom) shows the expression of the AR variants. C) Increasing hormone levels do not compensate for the diminished transcriptional response of E2K and P340L to ART-27. HeLa cells were transfected with 40 ng of each AR variant and 100 ng of ART-27 and 100 ng MMTV-luciferase. Cells were then treated with the indicated concentration of R1881 and luciferase activity determined as above.

Acknowledgement

References

Abagyan, R., and Argos, P. (1992). Optimal Protocol and Trajectory Visualization For Conformational Searches of Peptides and Proteins. Journal of Molecular Biology *225*, 519-532.

Abagyan, R., and Totrov, M. (1994). Biased probability Monte Carlo conformational searches and electrostatic calculations for peptides and proteins. J Mol Biol *235*, 983-1002.

Brinkmann, A. O. (2001). Molecular basis of androgen insensitivity. Mol Cell Endocrinol *179*, 105-109.

Buchanan, G., Greenberg, N. M., Scher, H. I., Harris, J. M., Marshall, V. R., and Tilley, W. D. (2001). Collocation of androgen receptor gene mutations in prostate cancer. Clin Cancer Res *7*, 1273-1281.

Castagnaro, M., Yandell, D. W., Dockhorn-Dworniczak, B., Wolfe, H. J., and Poremba, C. (1993). [Androgen receptor gene mutations and p53 gene analysis in advanced prostate cancer]. Verh Dtsch Ges Pathol *77*, 119-123.

Chamberlain, N. L., Whitacre, D. C., and Miesfeld, R. L. (1996). Delineation of two distinct type 1 activation functions in the androgen receptor amino-terminal domain. J Biol Chem *271*, 26772-26778.

Choong, C. S., Kemppainen, J. A., and Wilson, E. M. (1998). Evolution of the primate androgen receptor: a structural basis for disease. J Mol Evol *47*, 334-342.

Choong, C. S., Quigley, C. A., French, F. S., and Wilson, E. M. (1996). A novel missense mutation in the amino-terminal domain of the human androgen receptor gene in a family

with partial androgen insensitivity syndrome causes reduced efficiency of protein translation. J Clin Invest *98*, 1423-1431.

Culig, Z., Klocker, H., Bartsch, G., Steiner, H., and Hobisch, A. (2003). Androgen receptors in prostate cancer. J Urol *170*, 1363-1369.

Frishman, D., and Argos, P. (1996). Incorporation of non-local interactions in protein secondary structure prediction from the amino acid sequence. Protein Eng *9*, 133-142.

Frishman, D., and Argos, P. (1997). Seventy-five percent accuracy in protein secondary structure prediction. Proteins *27*, 329-335.

Fujimoto, N., Mizokami, A., Harada, S., and Matsumoto, T. (2001). Different expression of androgen receptor coactivators in human prostate. Urology *58*, 289-294.

Gelmann, E. P. (2002). Molecular biology of the androgen receptor. J Clin Oncol *20*, 3001-3015.

Gottlieb, B., Pinsky, L., Beitel, L. K., and Trifiro, M. (1999). Androgen insensitivity. Am J Med Genet *89*, 210-217.

Gregory, C. W., He, B., Johnson, R. T., Ford, O. H., Mohler, J. L., French, F. S., and Wilson, E. M. (2001). A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Res *61*, 4315-4319.

Gstaiger, M., Luke, B., Hess, D., Oakeley, E. J., Wirbelauer, C., Blondel, M., Vigneron, M., Peter, M., and Krek, W. (2003). Control of nutrient-sensitive transcription programs by the unconventional prefoldin URI. Science *302*, 1208-1212.

He, B., and Wilson, E. M. (2002). The NH(2)-terminal and carboxyl-terminal interaction in the human androgen receptor. Mol Genet Metab *75*, 293-298.

He, W. W., Fischer, L. M., Sun, S., Bilhartz, D. L., Zhu, X. P., Young, C. Y., Kelley, D. B., and Tindall, D. J. (1990). Molecular cloning of androgen receptors from divergent species with a polymerase chain reaction technique: complete cDNA sequence of the mouse androgen receptor and isolation of androgen receptor cDNA probes from dog, guinea pig and clawed frog. Biochem Biophys Res Commun *171*, 697-704.

Heinlein, C. A., and Chang, C. (2002). Androgen receptor (AR) coregulators: an overview. Endocr Rev *23*, 175-200.

Jenster, G., van der Korput, H. A., Trapman, J., and Brinkmann, A. O. (1995). Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem *270*, 7341-7346.

Jenster, G., van der Korput, H. A., van Vroonhoven, C., van der Kwast, T. H., Trapman, J., and Brinkmann, A. O. (1991). Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Mol Endocrinol *5*, 1396-1404.

Kasper, S., Rennie, P. S., Bruchovsky, N., Lin, L., Cheng, H., Snoek, R., Dahlman-Wright, K., Gustafsson, J. A., Shiu, R. P., Sheppard, P. C., and Matusik, R. J. (1999). Selective activation of the probasin androgen-responsive region by steroid hormones. J Mol Endocrinol *22*, 313-325.

Krongrad, A., Wilson, J. D., and McPhaul, M. J. (1995). Cloning and partial sequence of the rabbit androgen receptor: expression in fetal urogenital tissues. J Androl *16*, 209-212.

Lee, D. K., Duan, H. O., and Chang, C. (2000). From androgen receptor to the general transcription factor TFIIH. Identification of cdk activating kinase (CAK) as an androgen receptor NH(2)-terminal associated coactivator. J Biol Chem *275*, 9308-9313.

Li, P., Yu, X., Ge, K., Melamed, J., Roeder, R. G., and Wang, Z. (2002). Heterogeneous expression and functions of androgen receptor co-factors in primary prostate cancer. Am J Pathol *161*, 1467-1474.

Lu, B., Smock, S. L., Castleberry, T. A., and Owen, T. A. (2001). Molecular cloning and functional characterization of the canine androgen receptor. Mol Cell Biochem *226*, 129-140.

Markus, S. M., Taneja, S. S., Logan, S. K., Li, W., Ha, S., Hittelman, A. B., Rogatsky, I., and Garabedian, M. J. (2002). Identification and characterization of ART-27, a novel coactivator for the androgen receptor N terminus. Mol Biol Cell *13*, 670-682.

Mestayer, C., Blanchere, M., Jaubert, F., Dufour, B., and Mowszowicz, I. (2003). Expression of androgen receptor coactivators in normal and cancer prostate tissues and cultured cell lines. Prostate *56*, 192-200.

Nelson, W. G., De Marzo, A. M., and Isaacs, W. B. (2003). Prostate cancer. N Engl J Med *349*, 366-381.

Nemethy, G., Gibson, K. D., Palmer, K. A., Yoon, C. N., Paterlini, M. G., Zagari, A., Rumsey, S., and Scheraga, H. A. (1992). Energy parameters in polypeptides. 10. Improved geometrical parameters and nonbonded interactions for use in the ECEPP/3 algorithm, with application to proline-containing peptides. J Phys Chem *96*, 6472-6484.

Reid, J., Betney, R., Watt, K., and McEwan, I. J. (2003). The androgen receptor transactivation domain: the interplay between protein conformation and protein-protein interactions. Biochem Soc Trans *31*, 1042-1046.

Reid, J., Kelly, S. M., Watt, K., Price, N. C., and McEwan, I. J. (2002a). Conformational analysis of the androgen receptor amino-terminal domain involved in transactivation.

Influence of structure-stabilizing solutes and protein-protein interactions. J Biol Chem *277*, 20079-20086.

Reid, J., Murray, I., Watt, K., Betney, R., and McEwan, I. J. (2002b). The androgen receptor interacts with multiple regions of the large subunit of general transcription factor TFIIF. J Biol Chem *277*, 41247-41253.

Rogatsky, I., Zarember, K. A., and Yamamoto, K. R. (2001). Factor recruitment and TIF2/GRIP1 corepressor activity at a collagenase-3 response element that mediates regulation by phorbol esters and hormones. Embo J *20*, 6071-6083.

Stallcup, M. R., Kim, J. H., Teyssier, C., Lee, Y. H., Ma, H., and Chen, D. (2003). The roles of protein-protein interactions and protein methylation in transcriptional activation by nuclear receptors and their coactivators. J Steroid Biochem Mol Biol *85*, 139-145.

Tan, J. A., Joseph, D. R., Quarmby, V. E., Lubahn, D. B., Sar, M., French, F. S., and Wilson, E. M. (1988). The rat androgen receptor: primary structure, autoregulation of its messenger ribonucleic acid, and immunocytochemical localization of the receptor protein. Mol Endocrinol *2*, 1276-1285.

Taneja, S. S., Ha, S., Swensom, N. K., Pindea Torra, I., Rome, S., Walden, P. D., Huang, H. Y., Shapiro, E., Garabedian, M. J., and Logan, S. K. (2004). ART-27, an androgen receptor coactivator. J.Biol.Chem., in press.

regulated in prostate development and cancer. J Biol Chem.

Totrov, M. (2004). Accurate and efficient Generalized Born model based on solvent accesibility: Derivation and application for LogP Octanol/Water prediction and flexible peptide docking. J Comp Chem, in press.

Xu, J., and Li, Q. (2003). Review of the in vivo functions of the p160 steroid receptor

coactivator family. Mol Endocrinol *17*, 1681-1692.
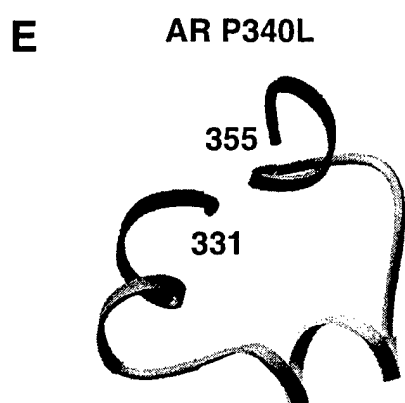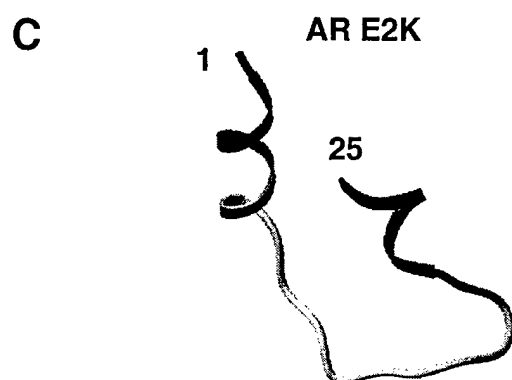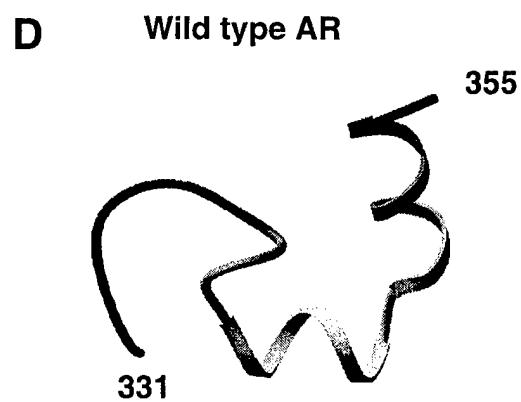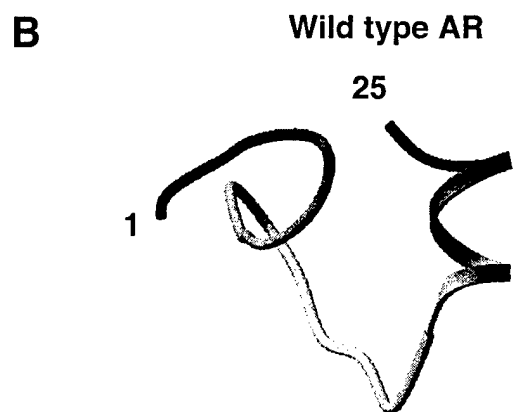
Figure 1

A

AR N — [Q] AF-1a AF-1b 500 DBD LBD AF-2 920 — C

1

AIS

E2K
*Q194R   N233K
*L255P
G491S

Q   1a   1b

PCa

50   100   150   200   250   300   350   400   450   500

K180R
E198G
E211K**

*M266T
P269S

S334P
P340L

A234T/
E236G**

- α-helix
- β-sheet
- random

B Wild type AR

25

1

C AR E2K

1

25

D Wild type AR

355

331

E AR P340L

355

331

Figure 2

A

(Bar chart: RLU on y-axis, ranging from 0 to 4000)

Legend:
- □ -R1881 ⌉ - ART-27
- ▨ +R1881 ⌋
- □ -R1881 ⌉ +ART-27
- ■ +R1881 ⌋

X-axis labels: VO, WT, E2K, K180R, Q194R, E198G, E211K, N233K, A234T/E236G, L255P, M266T, P269S, S334P, P340L, G491S

B

-ART-27 | α-AR   → AR
        | α-actin → actin

+ART-27 | α-AR   → AR
        | α-actin → actin

R1881 - + - + - + - + - + - + - + - + - + - + - + - + - + - +

C

(Bar chart: Fold Induction on y-axis, ranging from 0 to 60)

Legend:
- □ - ART-27
- ■ + ART-27

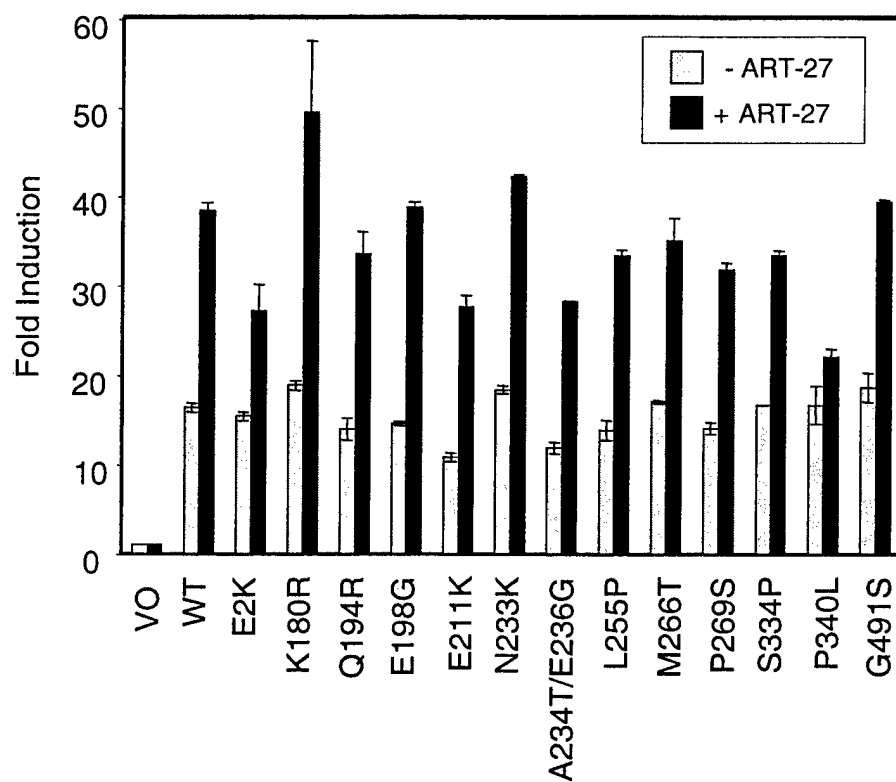X-axis labels: VO, WT, E2K, K180R, Q194R, E198G, E211K, N233K, A234T/E236G, L255P, M266T, P269S, S334P, P340L, G491S

Supplemental Figure 2C

Figure 3

Figure 4

A

B